

Supporting Information

Routes of iron entry into, and exit from, the catalytic ferroxidase sites of the prokaryotic ferritin *SynFtn*

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		B	TR	B	
SynFtn	:	SMNPDLLSAIQQHISIERIASVTVLAMSIIWCAE--REI	AGFYQFFDGEAK	DEQSHAVHFTQYLIARS	QSNDLQTLDAAPRQ-----NWDSL : 99
HumanH	:	NYHQDSEAAINRQINLELYASYVYLSMSYFFDRDDVAL	KNFAYFLHQSH	EREHAEKLMLQNQRGGRI	FLQDIKKPDC---DDWESG : 97
FrogM	:	NYHSDCEAAVNRLNLELYASYTYSSMYAFFDRDDVAL	HNVAEFFKEKSH	EREHAEKFMKYQNKRGGRVVLQDIKKPER---	DEWGNT : 93
HorseH	:	NYHQDSEAAINRQINLELYASYVYLSMSYFFDRDDVAL	KNFAYFLHQSH	EREHAEKLMLQNQRGGRI	FLQDIKKPDC---DDWENG : 97
RabbitH	:	~~~~~AINRQINLELYASYVYLSMSYFFDRDDVAL	KNFAYFLHQSH	EREHAEKLMLQNQRGGRI	FLQDIKKPEY---DDWESG : 78
MouseH	:	NYHQDAEAAINRQINLELYASYVYLSMSYFFDRDDVAL	KNFAYFLHQSH	EREHAEKLMLQNQRGGRI	FLQDIKKPDR---DDWESG : 97
Soybean	:	NYADECESAINEQINVEYNASYVYHSLFAYFDRDNVAL	KGFAKFFKESSE	EREHAELMKYQNTGRGRVVLHPKINAPSEFEHVEK	GDA : 173
Maize	:	KFVDDCEAAALNEQINVEYNASYAYHSLFAYFDRDNVAL	KGFAKFFKESSE	EREHAELMKYQNTGRGRVVLQSIPTLTFEFDHPEK	GDA : 174
Pea	:	NFADECESVINEQINVEYNASYVYHSLFAYFDRDNVAL	KGFAKFFKESSE	EREHAELMKYQNTGRGRVVLHPKIDVPSEFEHVEK	GDA : 171
Ecoli	:	MLKPEMIEKLEQMNLELYSSLLYQMSAWCSY--HTE	EGAAFLRRHAQ	EMTHMQRLFDYLTDTGNLPRINTVES	PFA-----EYSSL : 83
Cjejuni	:	MLSKEVVKLNEQINKEMYAANLYLSMSSWCYE--NSLD	GAGAFLEFAHAS	EESDHAKKLITYLNETDSHVELQEVKQPEQ----	NFKSL : 83
Hpylori	:	MLSKDIIKLLNEQVNMENSSNLYMSMSSWCYT--HSLD	GAGLFLFDHAA	EYEHAKKLIVFLNENNVVQVLTIS	ISAPHE-----KFEGL : 83
Vcholerae	:	MLSQAMVVEHLNEQINLEFFSSNLYLQMSAWCED--KGF	DGAAEFLRAHAV	EMQHMQRLLFTYVSETGALPILGATAAPRH-----	DFASL : 83
Ypestis	:	MLKKEMAQKLNEQLNLEFYSANLYLQMSAWCSD--KGF	DGAAFLKKHSQ	EMQHMERLFEYLSGTG	SMPVLGTITAPPV-----DFASL : 120

		3-fold	TR	B	B	4-fold	
SynFtn	:	ASLMATAFQMEADTTSSIQSVYALAE	ERNSDTRTTVFLLD	PLIEAQIQSE	QFAYLL	GRVKFANG--DP	TALLVIDNELRAGQTORG~~~~ : 182
HumanH	:	LNAMECALHLEKNVNQSLLELHKLA	TDKNDPHLCDFI	ETHYLN	QVKAI	RELGDHV	TNLRKMGAPESGLAEYLFDKHTLGDSDNES~~~~ : 183
FrogM	:	LEAMQALQLEKTVNQALLDLHKLA	TDKNDPHLCDFI	SEYLE	QVKDI	KRIGDFT	TNLRKMGAPESGMGEYLFDKHVSVESS~~~~ : 176
HorseH	:	LKAMECALHLEKNVNESLLELHKLA	TDKNDPHLCDFI	ETHYLN	QVKAI	RELGDHV	TNLRKMGAPESGMGEYLFDKHTLGDSDNES~~~~ : 182
RabbitH	:	LNAMECALHLEKSVNQSLLELHKLA	TDKNDPHLCDFI	ETHYLN	QVKSI	RELGDHV	TNLRKMGAPESGMGEYLFDKHTLGDSDNES~~~~ : 164
MouseH	:	LNAMECALHLEKSVNQSLLELHKLA	TDKNDPHLCDFI	ETHYLN	QVKSI	RELGDHV	TNLRKMGAPESGMGEYLFDKHTLGDSDNES~~~~ : 182
Soybean	:	LYAMELALSLEKLVNEKLLNVHVSVA	DRNNDPQMDDFIE	SEFLS	QVESIK	KISEYVA	QLRRVGK---GHGVVHFDQRLLD~~~~ : 250
Maize	:	LYAMELALSLEKLVNEKLLNVHVSVA	TRCNDPQLTDFIE	SEFLS	QGEAINK	KISKYVA	QLRRVGK---GHGVVHFDQRLLD~~~~ : 254
Pea	:	LYAMELALSLEKLVNEKLLNVHVSVA	ERNNDLEMTHTFIE	GEYLA	QVEAIK	KISEYVA	QLRRVGK---GHGVVHFDQRLLD~~~~ : 253
Ecoli	:	DELFTQETKHEQLITQKINELAHAA	MTNQDYPTFNFLQ	-WYVSE	QHEEEK	LFKSI	IKLVLGK--SGGLYFIDKELSTLDTQN~~~~ : 165
Cjejuni	:	LDVFETKYEHEQFITKINTLVHML	THKDYSTFNFLQ	-WYVSE	QHEEEA	LFRGIV	KIKLIGE--HGNGLYLADQYIKNIALSRKK~~~~ : 167
Hpylori	:	TQIFQKAYEHEQHISEINNIVDHA	IKGKDHTATFNFLQ	-WYVSE	QHEEEV	LFKDIL	KIELIGN--ENHGLYLADQYVKGIAKSRS~~~~ : 167
Vcholerae	:	GEVFRETYQHEQKITQINKLAHVA	FTSQDYSTFNFLQ	-WYVSE	QHEEEK	LFKGIL	DKLELVGE--DGKALFFIDKDLAALAKKGSSSV~ : 170
Ypestis	:	ADVFKLTYEHEQLITQINELAHVA	MTTHDYSTFNFLQ	-WYVSE	QHEEEK	LFKSI	IKLVLGN--SGNGLFFVDKDLKLTMAAQGYTSA~ : 206

Figure S1. Comparison of the sequence of SynFtn with those of selected other ferritins. The area shaded in blue denotes those residues that comprise the 3-fold channel with the positions of the two conserved carboxylates of the animal proteins marked by blue triangles. The area shaded orange denotes the residues that comprise the 4-fold channel. Red shading indicates residues that line the B-channel of prokaryotic ferritins and green shading the positions of the 'transfer carboxylates' identified in Frog M and Human H ferritin. The proteins shown are ferritin heavy chain from *Homo sapiens* (HumanH), ferritin middle subunit from *Rana catesbeiana* (FrogM), ferritin heavy chain from *Equus caballus* (HorseH), ferritin heavy chain from *Oryctolagus cuniculus* (RabbitH), ferritin heavy chain from *Mus musculus* (MouseH), ferritin 1 from *Glycine max* (Soybean), ferritin 1 from *Zea mays* (Maize), ferritin 1 from *Pisum sativum* (Pea), non-heme ferritin FtnA from *Escherichia coli* (Ecoli), non-heme ferritin from *Campylobacter jejuni* (Cjejuni), non-heme ferritin from *Helicobacter pylori* (Hpylori), ferritin from *Vibrio cholera* (Vcholerae) and ferritin from *Yersinia pestis* (Ypestis).

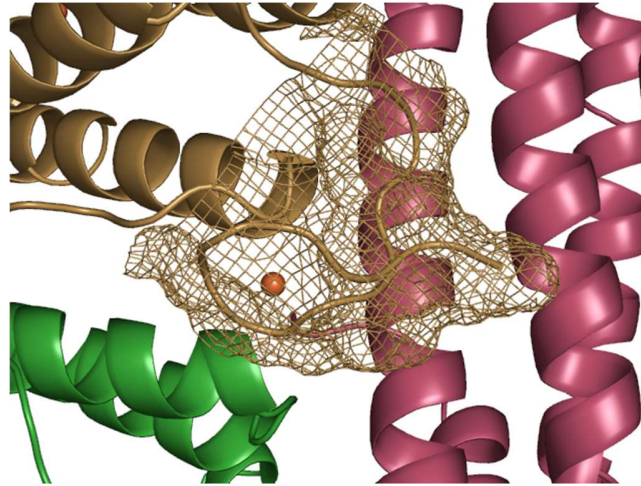


Figure S2. The B-channel of *SynFtn* is capped by an N terminal extension of the peptide chain. The image shows the B-channel of *SynFtn* formed at the intersection of three subunit monomers (PDB entry 6GKA) overlaid with an orange sphere at the position at which an iron ion was observed in the structure of E44Q *PmFtn* soaked overnight in a Fe^{2+} containing solution (PDB entry 4ZKH). The brown mesh represents the surface of the N terminal of *SynFtn*, which is an extension relative to *E. coli* FtnA, the most extensively characterized of the prokaryotic Ftn proteins.

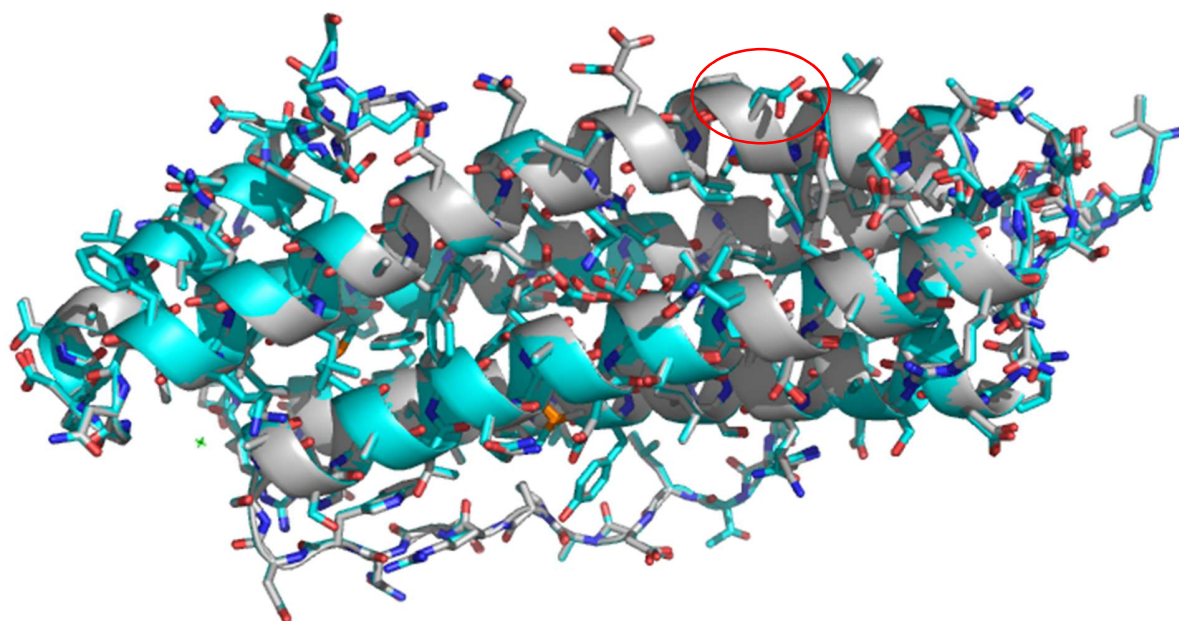


Figure S3. Comparison of the crystal structure of wild type and variant D137A *SynFtn*. Oxygen atoms coloured red, nitrogen blue, sulphur orange and carbon cyan (wild type) or white (D137A). The position of the mutated residue is marked by the red ellipse.

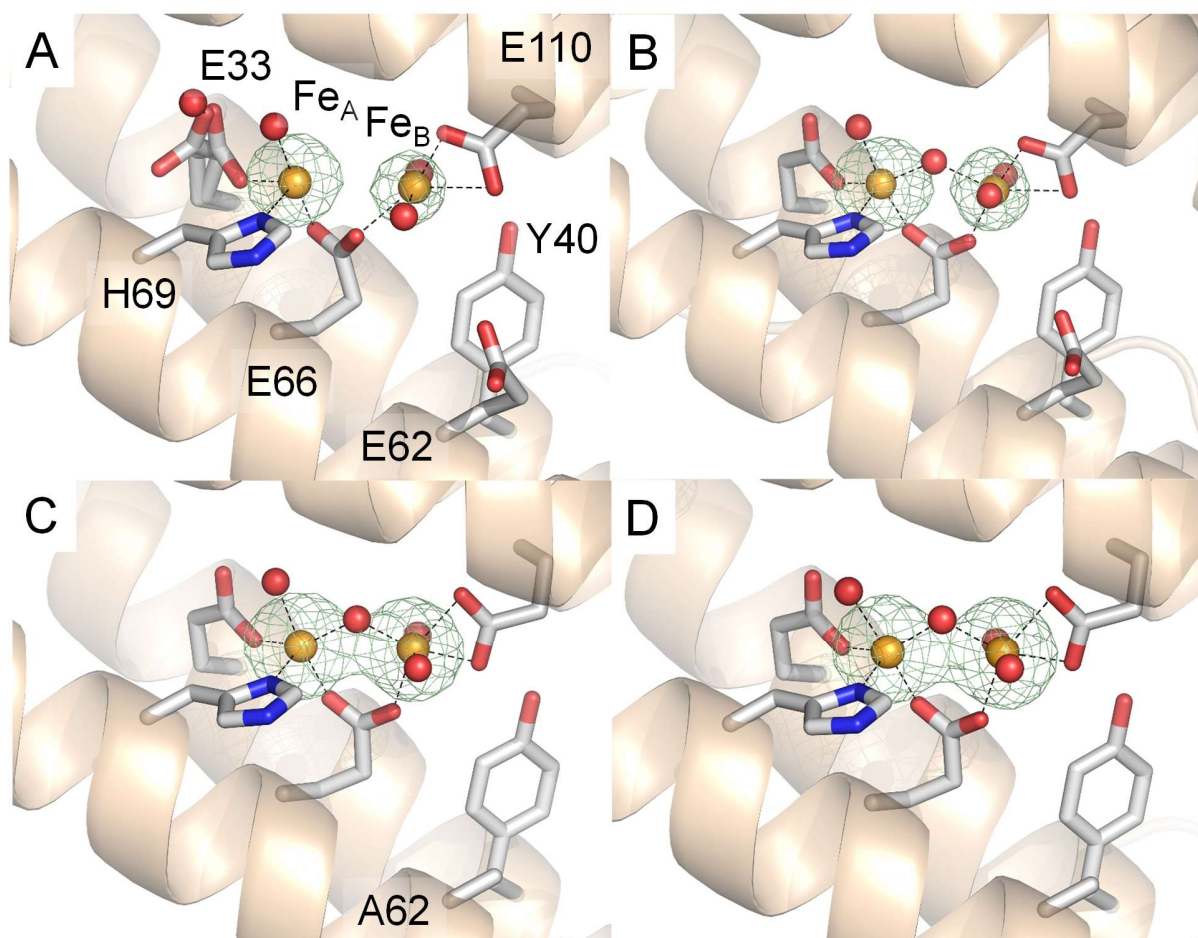


Figure S4. The ferroxidase center of Fe^{2+} -soaked SynFtn variants. The di-iron site of D137A SynFtn following the soaking of crystals in a 5 mM Fe^{2+} solution for either 2 min (A) or 20 min (B). (C-D) as (A-B) but for crystals of E62A SynFtn. Carbon is shown in white, nitrogen in blue, oxygen in red and iron as bright orange spheres with associated water molecules or (hydr)oxide ions as red spheres. The light green mesh in each of panels A to D shows the anomalous difference Fourier map calculated from data collected at the iron K-edge and contoured at 8σ .

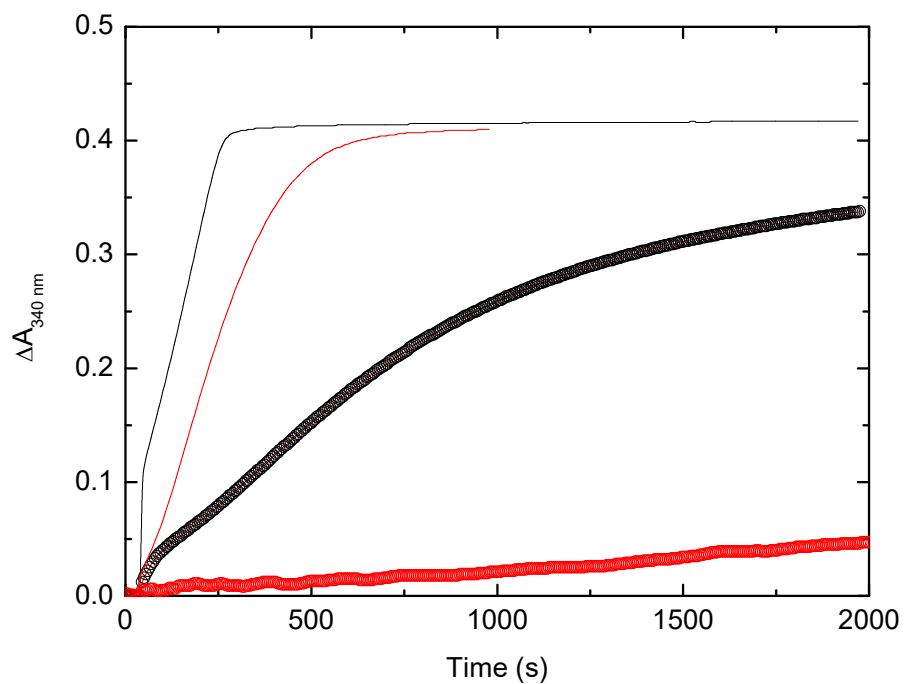


Figure S5. Inhibition of *SynFtn* by Zn^{2+} . The increase in absorbance at 340 nm as a function of time following the addition of 400 equivalents of Fe^{2+} to 0.5 μM protein (24mer). The response of wild type protein is shown in black and variant D137A in red, solid lines represent data for addition of Fe^{2+} to apo proteins and open circles for addition of Fe^{2+} to protein pre-incubated with 96 equivalents of Zn^{2+} (4 Zn^{2+} /monomer). All measurements were carried out in MES pH 6.5 at 25 $^{\circ}\text{C}$.

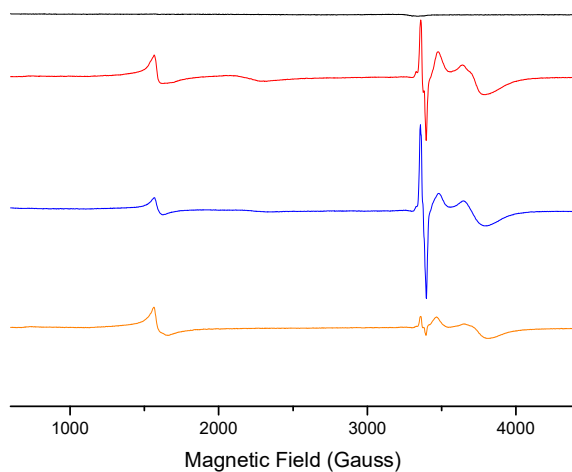


Figure S6. EPR properties of *SynFtn* and variants . Full sweep EPR spectra of: wild type *SynFtn* prior to the addition of Fe^{2+} (black); wild type *SynFtn* frozen 9 s after the addition of 72 equivalents of Fe^{2+} (red); variant E62A *SynFtn* frozen 12 s after the addition of 72 equivalents of Fe^{2+} (blue); and, variant D137A *SynFtn* frozen 20 s after the addition of 72 equivalents of Fe^{2+} (orange). Note that the intensity of the radical signal centred on 3386 G is variable between preparations and does not correlate with the E62A and D137A substitutions.

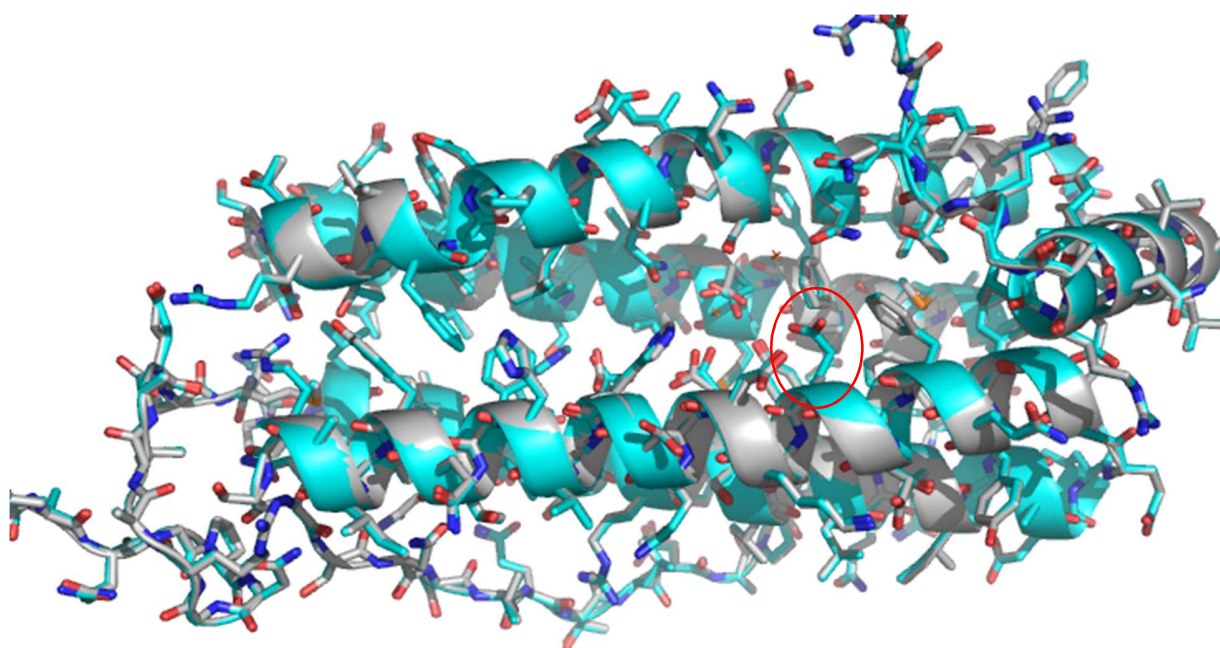


Figure S7. Comparison of the crystal structure of wild type and variant E62A SynFtn. Oxygen atoms coloured red, nitrogen blue, sulphur orange and carbon cyan (wild type) or white (E62A). The position of the mutated residue is marked by the red ellipse.

Table S1. *SynFtn* D137A mutant. Data collection and refinement statistics.

PDB code	6SOM	6SON	6SOO
Fe²⁺ Soak (min)	0	2	20
Wavelength	0.9763	0.9795	0.9795
Resolution range	40.55 - 2.15 (2.23 - 2.15)	51.07 - 1.60 (1.66 - 1.60)	44.12 - 1.57 (1.63 - 1.57)
Space group	F 4 3 2	F 4 3 2	F 4 3 2
Unit cell	176.8 176.8 176.8 90 90 90	176.9 176.9 176.9 90 90 90	176.5 176.5 176.5 90 90 90
Total reflections	223008 (22708)	609753 (61303)	852797 (83644)
Unique reflections	13408 (1302)	31808 (3112)	33428 (3301)
Multiplicity	16.6 (17.4)	19.2 (19.7)	25.5 (25.3)
Completeness (%)	99.81 (100.00)	99.93 (100.00)	99.96 (99.97)
Mean I/sigma(I)	10.25 (1.47)	26.10 (1.58)	27.06 (1.27)
Wilson B-factor	37.51	30.84	29.22
R-merge	0.1991 (1.465)	0.05239 (1.774)	0.06179 (2.264)
R-meas	0.2055 (1.509)	0.05386 (1.821)	0.06308 (2.31)
R-pim	0.05005 (0.3592)	0.01231 (0.4085)	0.01252 (0.4565)
CC1/2	0.997 (0.693)	1 (0.644)	1 (0.605)
CC*	0.999 (0.905)	1 (0.885)	1 (0.868)
Reflections used in refinement	13406 (1302)	31807 (3112)	33427 (3300)
Reflections used for R-free	677 (65)	1539 (153)	1671 (165)
R-work	0.1855 (0.2561)	0.1582 (0.2303)	0.1652 (0.2448)
R-free	0.2397 (0.3310)	0.1890 (0.2805)	0.1957 (0.2966)

CC(work)	0.957 (0.859)	0.968 (0.868)	0.959 (0.853)
CC(free)	0.946 (0.752)	0.948 (0.810)	0.956 (0.768)
Number of non-hydrogen atoms	1514	1630	1583
Macromolecules	1393	1416	1399
Ligands	1	3	2
Solvent	120	211	182
Protein residues	178	178	178
RMS(bonds)	0.006	0.005	0.005
RMS(angles)	0.71	0.69	0.67
Ramachandran favored (%)	98.86	100.00	100.00
Ramachandran allowed (%)	1.14	0.00	0.00
Ramachandran outliers (%)	0.00	0.00	0.00
Rotamer outliers (%)	0.00	0.66	0.00
Clashscore	1.83	1.79	0.73
Average B-factor	37.46	34.81	32.62
Macromolecules	36.95	33.02	31.18
Ligands	43.73	51.64	43.96
Solvent	43.35	46.58	43.56

Statistics for the highest-resolution shell are shown in parentheses.

Table S2. *SynFtn* E62A mutant. Data collection and refinement statistics.

PDB code	6SOP	6SOQ	6SOR
Fe²⁺ Soak (min)	0	2	20
Wavelength	0.9795	0.9795	0.9795
Resolution range	40.58 - 1.93 (2.00 - 1.93)	36.13 - 1.67 (1.73 - 1.67)	39.46 - 1.74 (1.80 - 1.74)
Space group	F 4 3 2	F 4 3 2	F 4 3 2
Unit cell	176.9 176.9 176.9 90 90 90	177 177 177 90 90 90	176.5 176.5 176.5 90 90 90
Total reflections	128714 (12505)	211108 (13333)	194099 (16168)
Unique reflections	18381 (1789)	27809 (2527)	24709 (2426)
Multiplicity	7.0 (7.0)	7.6 (5.3)	7.9 (6.7)
Completeness (%)	99.82 (99.89)	98.90 (91.85)	99.87 (100.00)
Mean I/sigma(I)	11.11 (1.19)	22.75 (1.44)	15.09 (1.52)
Wilson B-factor	35.55	28.17	30.79
R-merge	0.1016 (1.327)	0.04735 (0.9856)	0.06909 (0.9414)
R-meas	0.1098 (1.435)	0.05083 (1.093)	0.07412 (1.021)
R-pim	0.04087 (0.5372)	0.01811 (0.4579)	0.02631 (0.3896)
CC1/2	0.998 (0.49)	0.999 (0.496)	0.998 (0.581)
CC*	0.999 (0.811)	1 (0.814)	1 (0.857)
Reflections used in refinement	18379 (1789)	27808 (2526)	24708 (2426)
Reflections used for R-free	931 (90)	1398 (120)	1248 (119)
R-work	0.1550 (0.2735)	0.1511 (0.2555)	0.1575 (0.2304)
R-free	0.1998 (0.3269)	0.1830 (0.3370)	0.1873 (0.2812)

CC(work)	0.967 (0.802)	0.969 (0.810)	0.968 (0.844)
CC(free)	0.964 (0.755)	0.945 (0.645)	0.966 (0.836)
Number of non-hydrogen atoms	1583	1637	1600
Macromolecules	1395	1398	1395
Ligands	1	4	4
Solvent	187	235	201
Protein residues	178	178	178
RMS(bonds)	0.007	0.007	0.007
RMS(angles)	1.01	1.09	1.02
Ramachandran favored (%)	99.43	99.43	100.00
Ramachandran allowed (%)	0.57	0.57	0.00
Ramachandran outliers (%)	0.00	0.00	0.00
Rotamer outliers (%)	0.00	0.00	0.00
Clashscore	1.46	2.18	1.46
Average B-factor	37.11	30.13	33.13
Macromolecules	35.79	28.09	31.68
Ligands	39.32	30.23	32.88
Solvent	46.95	42.27	43.21

Statistics for the highest-resolution shell are shown in parentheses.

Table S3. Refined fractional occupancies of metal binding sites in *SynFtn* mutants. Soak solutions comprised the well solution with 5 mM Fe²⁺ and the pH adjusted to 6.5. Crystals were soaked for either 2 or 20 min prior to freezing.

<i>SynFtn</i> variant	Soak/ min	Fractional occupancy ^a		
		Site A	Site B	Site 3FC
D137A	2	0.50	0.32	0
	20	0.56	0.54	0
E62A	2	0.74	0.51	0.26
	20	0.92	0.88	0.33

^a Sites A and B constitute the ferroxidase centre. Site 3FC represents the iron binding site which lies on the symmetry axis in the 3-fold channel of the iron-soaked wild type ferritin structure (PDB entry 3OUY), and has a maximal occupancy of 0.33